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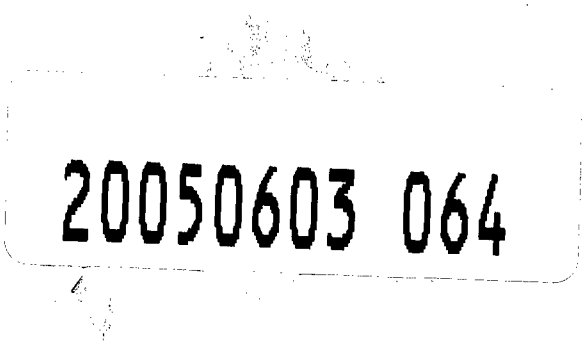

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In many breast cancer cells expression of the programmed cell death (apoptosis) promoting protein Bax is higher than in normal cells yet surprisingly the cells do not die. This observation suggests that one of the ways that breast cancers avoid undergoing apoptosis that would normally clear the tumor from the body at an early stage is by preventing Bax activation. Thus, we hypothesized that agents that specifically but inefficiently activate Bax would selectively eradicate breast cancer cells.

To test the hypothesis that activation of Bax would selectively induce apoptosis in breast cancer cells requires an agent that will act directly upon the Bax protein to activate it. To provide a reagent that would demonstrate proof-of-principle as well as provide a lead compound for drug discovery programs aptamers (small peptides or nucleic acids with high affinity and selectivity) were selected for different conformers of Bax by Covalent Display and SELEX. Covalent Display libraries of aptamers lacked sufficient diversity for the selection of high affinity aptamers. This was due to inefficient binding of P2A to DNA. Two other approaches to selecting aptamers were implemented and yielded promising preliminary results.

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Peptide aptamers as modulators of Bax mediated apoptosis in breast cancer cells

Introduction:

In many breast cancer cells expression of the programmed cell death (apoptosis) promoting protein Bax is higher than in normal cells yet surprisingly the cells do not die. This observation suggests that one of the ways that breast cancers avoid undergoing apoptosis that would normally clear the tumor from the body at an early stage is by preventing Bax activation. Thus, we hypothesized that agents that specifically but inefficiently activate Bax would selectively eradicate breast cancer cells.

To test the hypothesis that activation of Bax would selectively induce apoptosis in breast cancer cells requires an agent that will act directly upon the Bax protein to activate it. To provide a reagent that would demonstrate proof-of-principle as well as provide a lead compound for drug discovery programs requires an agent that will bind tightly to specific conformers of Bax. Such agents would then shift the equilibrium of conformers to the more active form. In this project aptamers (small peptides or nucleic acids with high affinity and selectivity) were selected for different conformers of Bax by Covalent Display and SELEX, respectively.

In Covalent Display peptide aptamers are selected using a library of peptides fused to either the amino- or carboxyl-terminus of a DNA binding protein (such as P2A) that binds to its own DNA template in cis after in vitro transcription translation. Recently a second protein called RepA has also proven useful for covalent display. Aptamers are selected using immobilized protein (of known conformation) and panning. Bax proteins in both the inactive and active conformation were prepared and characterized.

Both P2A and RepA libraries were constructed. Surprisingly P2A libraries lacked the required diversity for selection of high affinity aptamers. The reason the diversity in P2A libraries was lower than expected was found to be inefficient binding of P2A protein to DNA in vitro. RepA libraries are currently being analyzed for diversity and selection of aptamers.

In our application of SELEX, libraries of single stranded DNAs are immobilized by specific binding to a substrate on beads. This binding reaction is adjusted such that the affinity and off-rate match the desired affinity of the aptamer for Bax. Nucleic acid aptamers that bind to Bax are eluted from the beads by incubation in excess Bax.

In both approaches selection and amplification are repeated sequentially until aptamers with the desired characteristics are isolated. The results of selections of nucleic acid aptamers for Bax suggests the libraries contain sufficient diversity and that aptamers were being enriched however, insufficient cycles of selection were performed to obtain high affinity aptamers.

A cell free assay was established and validated that will be used for preliminary functional characterization of any aptamers selected in the future.

The selected aptamers will be used to validate Bax as a drug target for the successful treatment of breast cancer. These aptamers will also be useful lead compounds for drug discovery programs designed to identify new therapeutics for the treatment of breast cancer.

Body of Report

Task 1

Assembly of Covalent Display Libraries and selection of aptamers:

1-a.) Assembly of random libraries. We proposed to select peptide aptamers (peptides from random libraries that bind tightly to ligands) that bind to specific conformations of Bax to test the hypothesis that these aptamers will activate Bax translocation to mitochondria and/or that induce the Bax conformational change associated with oligomerization and commitment to cell death. Aptamer selection depends on the design of selection schemes that permit isolation of the appropriate peptides from Covalent Display libraries of 10^{10} or more peptides. Covalent Display libraries consist of semi-random DNA sequences that encode a semi-random distribution of an arbitrary number of amino acids as a fusion protein with the DNA binding protein P2a (Fitzgerald, 2000; Reiersen et al., 2005).

Task 1-a.1) 5' Library by primer extension. The first library was assembled at the 5' end of the P2a coding region by the method outlined in Figure 1. In the first step a linker was added to the 5 prime end of a PCR product encoding the DNA binding protein P2a. A second PCR product (Sequence 1) was created containing the library, a TAC promoter sequence and at the 3 prime end, a sequence matching the linker on the PCR product encoding P2a. The library and 3 prime primer PCR product was used as a megaprimer to amplify the P2a coding region generating the desired P2a peptide library fusion protein.

Sequence 1: Library and 3 prime primer

```
   1  cgggaaaaaa ttcacgtatc cggtcgctga tgagtggccg ggattctcaa
  51  cagtaatgga gtggacagga ggaagtggtg gaagtggaag tggtnnbnnb
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101  nnbnnbnnbn nbnnbnnbnn bnnbnnbnnb ggtagtagtg gatccggtta
151  gccgggcccg cagac
```

The randomized region is bold and underlined. The nnb semi-random coding scheme was used for all of the libraries in this project and includes codons for all 20 amino acids in roughly the normal frequency (positively charged residues are somewhat favoured) but contains only one of the three possible stop codons. As a result, one out of every 20 codons in the initial library should be a stop codon. Any sequences with a stop codon do not synthesize the P2a product required to link the DNA to the library. Therefore, one way to estimate success in early rounds of selection is to measure the rate at which stop codons disappear from the library. The library was amplified after each round of selection allowing a sample to be cloned into the vector psputk and sent for sequencing while the remainder was used for the next round of selection.

Task 1-a.2) 3' Library. This library was constructed using primer extension. Although this method appears similar to that used to generate the 5' prime library more diversity is preserved because there are fewer PCR cycles after adding the randomized sequence (See Figure 2). To generate this library a glycine/serine encoding linker was added to the full length P2a coding region by PCR. Addition of the linker also removed the stop codon from the P2a coding region. To this a primer containing the library with an additional unique extension after the library (and after the end of the coding region) was added. The extension included a unique restriction site to facilitate cloning the selected sequences and was used to amplify the complete sequence (eventually this required 20 cycles of amplification, see below). The 3 prime end was then amplified and

reassembled with the remainder of the 5 prime P2a PCR product by stitching the two PCR products together with external primers. After a second round of selection against Bax, the 3 prime end was amplified and sequences were cloned into a plasmid (psputk) and sent for sequencing.

Task 1-a.3) Cloning of B1 domain of protein A and protein L to use as scaffolds for the presentation of peptide libraries. The B1 domains of protein A and protein L were cloned by PCR and the coding regions were fused to the coding region for P2a. Expression in E. coli lysate of the fusion proteins was demonstrated using 35 SMet labelling and using western blotting. The synthesis of the B1 domain of protein L fused to P2a was particularly efficient as judged by western blotting (data not shown). Libraries of random sequences were not introduced into the exposed loop region of protein A and protein L due to difficulties with the efficiency of P2a binding to DNA (Task 1-c.2).

Task 1-b.1) Purification of Bax and Bid. The Baxα protein was expressed in E. coli using a Baxα-Intein expression vector and purified using the Impact system from New England Biolabs (Yethon et al., 2003). The final step in purification was a phenyl-Sepharose column which reduced the impurities sufficiently that the protein could be used to isolate aptamers (see Figure 3). Bid was purifed by expression in E. coli of a construct encoding full-length Bid with a His tag at the amino-terminus. After affinity chromatography on Nickel resin pure Bid was obtained (Figure 4). However, this protein is relatively inactive in activating Bax. Therefore, to convert Bid to the more active tBid (Figure 5A) the Bid protein was cleaved using His-tagged recombinant caspase 8 (see

Figure 5B). Once Bid was cleaved by incubation with caspase 8 for 3 hours (to minimize non-specific degradation) it was still necessary to separate the two fragments (tBid and p7) because the p7 fragment inhibits tBid. It was also necessary to remove the recombinant caspase 8. Both polypeptides were removed by passing the cleavage reaction over the nickel column a second time and eluting with 1.2% octylglucoside (OG) instead of imidazole. As shown in Figure 5C, tBid was efficiently eluted (the migration of the band is artificially altered by the addition of the detergent as it competes with SDS for binding to the protein) while p7 and residual full length Bid remained bound to the beads and were eluted with 250 mM Imidazole. Thus both Bax and tBid were purified on schedule in the quantities and purity needed for selection experiments.

Task 1-b.2) Assessing the conformation of the Bax molecules used for selections.

We expected that two positive selections and one counter selection should have been sufficient to eliminate all of the fusion proteins in which the library sequence encoded a stop codon (20 fold). Instead only 1/3 fold enrichment was obtained (see below Task 1-c.). Inefficient binding of the fusion protein to DNA may account for the low enrichment obtained because any carry over of the 10,000 fold excess DNA (not bound to a P2a fusion protein) will compete with bona fide binding complexes in the PCR reaction. This problem is further exacerbated because a large number of PCR cycles are required to obtain > 10,000 copies of each selected DNA (required to maintain a selected sequence), (see below, Task 1-c.). Thus, unless selection is very stringent the SELEX process will select for DNAs that amplify well by PCR rather than ones that encode a bona fide

binding partner. In addition, for selections to be stringent the Bax protein being used must adopt a single conformation. Therefore, another possible explanation for the relatively poor recovery from the selections was conformational heterogeneity in the Bax protein used as a target.

Task 1-b.3) Verify that Bax retains the correct conformation on solid supports using the conformation specific monoclonal antibody (6A7). To verify that Bax changed conformation only after exposure to Triton and not when adsorbed to tubes for selections we incubated the adsorbed protein with the conformation specific monoclonal antibody 6A7 linked to HRP. Colorimetric visualization of antibody binding confirmed that most Bax molecules remained 6A7 negative unless Triton was added. To determine the conformational diversity in our purified protein samples more precisely than has been possible using conformation specific antibodies we also used fluorescence spectroscopy.

Site directed mutagenesis was used to produce Bax mutants each with a unique cysteine residue (at amino acid positions 40, 66,79, 98,106, 138, 164 and 188). Mutagenesis was accomplished using the stitch together method due to the size of the Bax-intein plasmid expression vector. This method requires the isolation and sequencing of a number of colonies due to the possible alteration of sequences at other points of the molecule resulting from the error rate of the Taq polymerase. All mutants were created and expression and purification was as previously (see Task 1-b.1) except the Impact expression system was modified to use a cell line with an arabinose inducible T7 polymerase rather than induction using IPTG. The single cysteine mutants were labelled

with the environment sensitive dye NBD and assayed for conformational changes using fluorescence spectroscopy. Residue L38 proved useful for assessing conformational changes (Figure 6) and was therefore used to verify that selections used a single conformer of Bax.

Task 1-b.4) Biotinylate Bax for recovery on immobilized streptavidin. Single cysteine mutants of Bax described in Task 1-b.2) were biotinylated using maleimido-biotin. The cysteine located at residue 40 was most efficiently biotinylated and could be recovered using streptavidin agarose. This protein was not further used in selections due to problems with the efficiency of P2a DNA binding (Task 1-c.2).

Task 1-c. Selection, amplifying and cloning of PCR products.

Task 1-c.1) Selection Methods. Selections were performed using purified Baxα protein. Counter selections used Baxα protein that was activated using non-ionic detergent. Therefore these aptamers should bind specifically the non-active form of Bax. The covalent display library was expressed by in vitro transcription translation using an E.coli S30 lysate system prepared in house. The in vitro transcription translation reactions were 50 ul reactions in which 1 ug of PCR produced Library-P2a was expressed for 30 min. at 30 ° C. Reactions were diluted with PBS before being added to immunoreaction tubes previously coated with Bax or Triton X-100 activated Bax protein and blocked with Pierce blocking buffer. After 1 to 2 hours of incubation at room temperature the unbound material was removed and the tubes washed 3 times with PBS.

The bound DNA-peptide-library-P2A was extracted with 10 mM TRIS pH 8.0, 1% SDS and proteinase K. The DNA was isolated using silica matrix binding, the library

containing region was amplified by PCR and then used for the next round of selection. At each assembly stage the 5 prime end primer had additional sequences extending the molecule to allow for further rounds of amplification without primer fatigue.

PCR products from the second round of selection as well as the products bound to the counter selection of activated Bax (In Figure 7, selection on Bax followed by counter selection with Triton activated Bax is designated BT) were used to clone some sequences to evaluate the progress of the selections. DNA sequences were cloned in the vector pGem7Zf and plasmid DNA from selected clones was minipreped and checked for inserts. Plasmids with inserts were then sent for sequencing. As shown for BT and BTB (selection Bax, counter-selection Triton-Bax, selection Bax) selected sequences (Figure 3) only one pair of sequences was identical, there was no similarity in the sequences suggesting that there is still diversity in the library but there was an unexpectedly high number of stop codons. Furthermore, when selection was continued several more rounds we discovered that we began accumulating sequences without inserts suggesting we were losing the library due to more efficient amplification of contaminating PCR product or plasmid for P2A. That result suggested an unexpected loss in diversity when selecting aptamers for Bax that we had not seen in control selections previously that were doped with 'correct' sequences (data prior to submission of the grant application).

Nevertheless, the decrease in the number of stop codons from the expected 1:20 to 1:27 suggested that the selection system was working but that another factor was responsible for the loss in diversity. This necessitated a change in strategy as it was necessary to identify and correct the source of this problem. Two possible reasons for the

problem were investigated: 1) The efficiency of binding of the P2a-library to DNA was too low. 2) The proteins used to select the aptamers were not conformationally unique.

Task 1-c.2) The P2a-library fusion protein binds to DNA with low efficiency.

To evaluate binding of the Library-P2a fusion protein to its encoding DNA in cis, a library was added to P2a similar to above. However, in this case in the final assembly PCR the 5 prime primer was made containing a terminal biotin. The biotin incorporated into the DNA used as a template for in vitro transcription translation and thereby allowed us to isolate the biotin labelled DNA on a streptavidin coated ferric beads using a magnet. The amount of P2a fusion protein bound to the isolated DNA was assayed quantitatively by measuring the amount of the protein synthesised in the in vitro transcription translation reaction that bound DNA based on the incorporation of 35S-met into the protein. The protein bound to the beads was quantified by scintillation counting while unbound protein was assayed after acid precipitation by scintillation counting. The specific activity of the 35S-met was used to estimate that number of molecules of protein bound to DNA. The ratio of bound protein to DNA was found to vary depending on the amount of DNA added to the reaction suggesting that we were using more than saturating amounts of DNA. More concerning, we discovered that in our standard conditions approx. 1 of 10,000 DNA molecules of the PCR product encoding the Library-P2a fusion protein had a Library-P2a protein bound. Attempts to improve this by altering the salt concentrations in the S30 transcription translation reaction were unsuccessful (see Table One).

	conc. DNA	CPM	DPM	molecules of P2A bound (x1,000,000)
Standard	3ug	20858	22260	216.6
reaction	1ug	12070	12870	125.4
	.3ug	6420	6844	66.6
	.1ug	11193	11949	116.4
50mM NH4	3ug	11257	12017	117
12mM Mg	1ug	6567	7008	68.4
	.3ug	7573	8132	79.2
	.1ug	11754	12628	120
50mM NH4	3ug	10996	11745	196
	1ug	14427	15405	150
	.3ug	6516	6948	67.8
	.1ug	8144	8712	84.6
RNase	1ug	42102	46324	450.6
	1ug	43401	46471	452.4
Background	0ug	1474	1578	

Table One: Binding of P2a fusion proteins to biotinylated PCR products.

Biotin labeled PCR product was used standard and modified ITT reactions containing additional NH_4 or Mg salts.
The reaction contained variable amounts of the PCR product (3, 1, .3, .1 ug).
Transcription translation was carried out at 37° C for 30min. in reactions containing 1nmol/ml ^{35}S-methionine
The reaction was diluted with buffer A 1.5-fold (45ul), for RNase treatment on ice for 10 min
20ul of paramagnetic beads were added and incubated on ice 30 min.
Beads were washed 3 times with buffer A and 3 times with RIPA and resuspended in 100ul TBS.
Radioactivity was measured in scintillation counter
DPM are used to calculate the number of molecules bound to the biotin labelled PCR DNA.

As the assembly of the final library-P2a involved amplification by 12 cycles of PCR each library member was represented on average 2^{12} or approx. 4000 times. If only 1 in 10,000 DNAs is bound to a P2a protein (as our data indicated) then we estimate that there was approximately a.2.5 fold loss of diversity in the first round due to insufficient amplification. To compensate for the inefficient utilization of the template DNA, additional rounds of amplification of the PCR product encoding the library fused to P2a were added (up to 16 to 20 cycles) in the next library.

Task 1-c.3 Assembly of additional random libraries (extension of Task 1a.)

In an attempt to increase the efficiency with which the P2a fusion protein bound to DNA, a library was constructed as a fusion to the carboxyl-terminus of P2a. Unfortunately these fusion proteins also bound to DNA inefficiently. To determine whether Bcl-2 binding peptides interfere with P2A binding to DNA as well as measure what copy number in the library is required to select a Bax binding peptide from the library a peptide encoding the BH3 region of Bid was added in place of the library. The efficiency of DNA binding and selection is being assayed for this construct.
In addition, it was discovered that DNA biding is more efficient for a Rep A fusion protein than for P2a (Odegrip et al., 2004). Therefore this protein is being tested in our system to determine it is more appropriate than P2a for selecting Bax binding aptamers.

To accomplish this, a plasmid was located at ATCC, (plasmid pDR31) which contained the sequence required to recreate this display system. Whereas a previous publication with this method used a ligation step followed by a stitch together PCR step to create and amplify the library we decided to clone a majority of the RepA into a plasmid, pCfive-1. This prevents the inadvertent introduction of mutations into a majority of the coding sequence including the promoter. In addition the library being in the expression vector allows the cloning at any step, sequencing and expression of the peptide at high levels which will be useful for screening in the above mentioned techniques.

To validate this system we doped the library with sequences encoding Bim and Bim mutant peptides. These DNAs were created similar to the library. Selective recovery

of DNAs encoding the Bim peptide (and not the Bim mutant peptide) will verify that the selection system is working. This work is on going and the third round of selection has been completed and the library is being cloned out and individual clones sent for sequencing.

Conclusions – Task 1. In total 5 random libraries were created. Both proteins required for selections were purified successfully. While two of the three selections completed resulted in partial elimination of inappropriate aptamers the diversity in the library was insufficient to select aptamers of sufficient affinity and specificity to use in Task 3. The results of the third selection are presently being quantified. However, due to an unexpected loss in diversity in the libraries during selection, appropriate aptamers required for Task 3 were not selected prior to the termination of funding for this project.

Task 2.

Verification of putative Bax binding peptides in vitro.

Task 2-a.) Obtain synthetic Bax binding peptides. Because aptamers of sufficient affinity were not obtained on schedule we obtained synthetic Bax binding peptides based on the BH3 domain sequences of the Bax interacting proteins tBid and Bim. These peptides served as surrogate aptamers to permit completion of Task 2-b. The required peptides and matched peptides with inactivating point mutations were purchased from Dalton Chemical Company.

Task 2-b.) Test the effect of peptides on Bax translocation to mitochondria and on Bax conformation in vitro. The tBid and Bim BH3 peptides allowed us to continue the project and set up assays to test the effect of peptides using a cell free

system. In this way the assay system would be prepared and characterized so that when aptamers were available they could be tested immediately.

To assay Bax and the peptides we used a cell free system in which the extent of cytochrome c that was released from mitochondria by Bax after activation with tBid (as a positive control) or BH3 peptide was assayed by western blotting. Heavy membranes enriched in mitochondria were isolated essentially as in (Eskes et al, 2000) from cells lysed by nitrogen cavitation at 150 psi for 15 min on ice in a 45 mL Nitrogen Bomb (Parr Instrument) (Annis, 2001). The debris was separated from the lysate by centrifugation at 2,000 x g for 4 minutes. Mitochondria and other heavy membranes were isolated from the supernatant by centrifugation at 13,000 x g for 10 minutes at 4°C. The heavy membrane pellet was resuspended in MB (10mM HEPES pH 7.5, 150mM KCl, 210mM mannitol, 70mM sucrose, 0.5mM EGTA) and used immediately.

The isolated heavy membranes were diluted to a concentration of 1mg/mL of mitochondrial proteins with MBC buffer (210mM mannitol, 150mM KCl, 0.5mM EGTA, 10mM HEPES pH 7.5 4mM $MgCl_2$, 5mM Na_2PO_4, 5mM succinate and 5μM rotenone) and incubated with tBid or BH3 peptide for 1 hour at 30°C. Mitochondria were pelleted by centrifugation at 13,000 x g for 10 minutes and the amount of cytochrome c in the supernatants and pellets was analyzed by SDS-PAGE and immunoblotting using nitrocellulose membranes.

To characterize this cell free system for the analysis of Bax activation we used mitochondria isolated from HOMyc3 cells (Kim et al. 2004). A small amount of cytochrome c was released from the HOMyc3 mitochondria after exposure to 0.83 nM

tBid, and was increased when 1.67 nM tBid was added (Figure 8A). Since these mitochondria do not contain detectable Bax but do contain Bak, cytochrome c release was likely due to tBid activation of Bak. When 0.42 nM tBid was added with 104 nM Bax most of the cytochrome c was released (Figure 8A, B). Because 0.42 nM tBid released cytochrome c only when Bax was added we conclude that cytochrome c release was primarily due to activation of Bax (rather than Bak) by tBid. To determine that this assay could also be used to estimate the specific activity of Bax added to the reactions the assay was repeated with a fixed amount of tBid (insufficient alone to release cytochrome c) and Bax was titrated into the reactions. As can be seen in Figure 8B the amount of cytochrome c released is directly proportional to the concentration of added Bax confirming that this system is an appropriate assay for Bax activity. Identical results to those shown in Figure 8A were obtained when BH3 peptides from Bim or Bid were titrated into reactions containing Bax, confirming that this assay can be used to assay any aptamers selected. To make this assay more quantitative mitochondria were isolated from cells expressing Bcl-2 as well as from control cells transfected with an empty vector. Data for Bim BH3 peptide activation of Bax is shown in Figure 9.

Task 2-c. was not attempted because aptamers were not obtained that were demonstrated to function in the in vitro assay.

Task 2 – Conclusions The data in Figures 8-9 demonstrates that Task 2-a. and 2-b. were completed on time.

Task 3.

Test the activity of Bax binding aptamers to restore apoptosis in or sensitize breast cancer cells to chemotherapy drugs.

Due to difficulties completing Task 1-c., Task 3 was not attempted.

Replacement Task 3. Instead of the originally scheduled Task 3 **we continued Task 1-c.** in a more profitable direction. The primary challenge in this project was selecting aptamers for different conformers of Bax. Since we were unable to do this on schedule by Covalent Display we attempted selection of aptamers using another selection system. In this system single stranded DNA aptamers are selected from libraries of 10^{12} sequences. An example of the data from a single round of selection is provided in Figure 10. Selections were carried out for A) Soluble Bax (inactive) counter selected against Bax activated using a Bid BH3 peptide, B) Soluble Bax activated using a Bid BH3 peptide counter selected against Soluble Bax (inactive), C) Membrane bound Bax (incubated with BH3 peptide and liposomes) counter selected against soluble Bax, D) Soluble Bax counter selected against membrane bound Bax. BidBH3 peptide was used to activate Bax rather than using Triton (as above) because we were able to show that peptide activation gave us the conformational homogeneity required for selections (Task 1-b.). As expected the yield of potential aptamers increased dramatically at each cycle of selection (selection results are summarized in Figure 11). For A) 6 rounds of selection for soluble Bax counter selected against activated Bax were completed. Enrichment was convincingly detected in round 5 where ratio of the number of aptamers selected relative to negative selection (control) was 1.4. After round 6 this ratio had increased to 7.1 suggesting that we have obtained an initial population of aptamers. For B) 5 rounds of

selection were completed. The relative enrichment for aptamers specific for activated soluble Bax was 4.6. For C) 4 rounds of selection were completed. The enrichment for aptamers specific for membrane bound Bax counter selected against soluble Bax was 4.5. The only selection that was not successful in selecting aptamers was the selection for soluble Bax with counter selection for membrane bound activated Bax. In this case 5 rounds of selection resulted in an 'enrichment' of only 1.02. While 3 of the 4 selections gave favourable results, insufficient rounds of selection were completed before the funding period ended to select aptamers of sufficient affinity and specificity to use in Task 3.

Task 3 – Conclusions: Aptamers of sufficient quality for testing for effects on breast cancer cells were not obtained prior to termination of funding. However, the extension of Task 1-c. carried out during the time allotted for Task 3 was successful in generating aptamers to Bax. Further optimization of these aptamers is required before they can be tested in either cell free or cell based systems for efficacy in inducing programmed cell death in breast cancer cells.

Key Research Accomplisments:

1) Random libraries of PCR products encoding aptamer sequences fused to the 5' and 3' end of P2a were generated and verified by DNA sequencing.

2) Bax, Bid and tBid were expressed and purified in sufficient quantities for screening libraries.

3) Bax was immobilized on immunosorb tubes and magnetic microspheres for use in screening libraries.

4) A single cysteine mutant of Bax was identified that can be efficiently biotinylated for use in selections.

5) B1 domains of protein L and protein A were cloned and the coding regions were fused to the coding region for P2a. Expression of the fusion proteins in S30 lysate was verified.

6) PCR products encoding putative Bax binding peptide aptamers were selected, amplified, sequenced and cloned. Selection revealed an inherent inefficiency in P2a DNA binding that limited diversity in libraries.

7) Diversity in the libraries was analyzed by DNA sequencing of the PCR product directly and by cloning individual genes from the library and sequencing them (Figure 7).

8) The effect of Bax binding peptides on Bax translocation to mitochondria and Bax conformation was assessed using purchased peptides corresponding to BH3 domains of Bim and Bid.

9) A replacement peptide aptamer selection system using RepA instead of P2a was assembled and preliminary testing was carried out.

10) An alternative aptamer selection system using single stranded DNA aptamers was implemented.

11) Single stranded DNA aptamers that bind to three of four conformations of Bax were enriched in libraries by selection in vitro. These libraries can be further selected to yield aptamers that can be used to test the original hypothesis that aptamers can alter Bax function in breast cancer cells.

Reportable Outcomes:

1) Peptide libraries fused to RepA that can be used to select aptamers to proteins have been created and stored.

2) Bax, Bid and tBid proteins have been purified and can be used in other studies.

3) An assortment of single cysteine mutants of Bax that can be labelled using maleimide derivatives have been generated (plasmids and purified protein).

3) Funding has been applied for from CIHR to use the single cysteine mutants of Bax in fluorescence studies of Bax conformation changes.

4) Steve Primorac has been hired to work on a Genome Canada project that involves selection of single stranded DNA aptamers with high affinity for proteins (including Bax) based on his work with aptamer libraries and protein purification in this project.

Conclusions:

This research project was the first practical application of Covalent Display as a technique for the selection of peptide aptamers. We discovered that the efficiency with which P2a binds to DNA in cell free lysates is not sufficient to permit selection of aptamers. However, it appears that it is possible to select aptamers for Bax using libraries of single stranded DNAs. Furthermore, preliminary data suggests another protein RepA appears to bind to DNA with sufficient efficiency to compete with the selection of plasmids that do not encode aptamers but are more efficient substrates for PCR. Thus, evidence was generated suggesting that two alternate approaches may provide aptamers specific for Bax conformers. These methods for selecting aptamers

need to be further investigated to demonstrate their utility for future work designed to isolate conformation specific aptamers for Bax.

It was not possible to test the hypothesis that aptamers can shift the conformational equilibrium for Bax to sensitize breast cancer cells to programmed cell death. However, experiments using BH3 peptides confirmed that it is possible to manipulate the conformation of Bax using peptides. Furthermore, we demonstrated that fluorescence changes of NBD labelled Bax can be used to monitor conformational changes in the protein. Together these results suggest that the NBD-labelled single cysteine mutants of Bax created here can be used as the basis for a high-throughput screen for small molecules that will activate Bax. If such molecules can be found they would represent lead compounds for agents that would induce cell death selectively in breast cancer cells.

List of Personnel (supported in part or in whole by this grant): Steve Primorac, Xiumei Yang, Lieven Billen (supplies only), Paulina Dlugocz, Jon Lovell.

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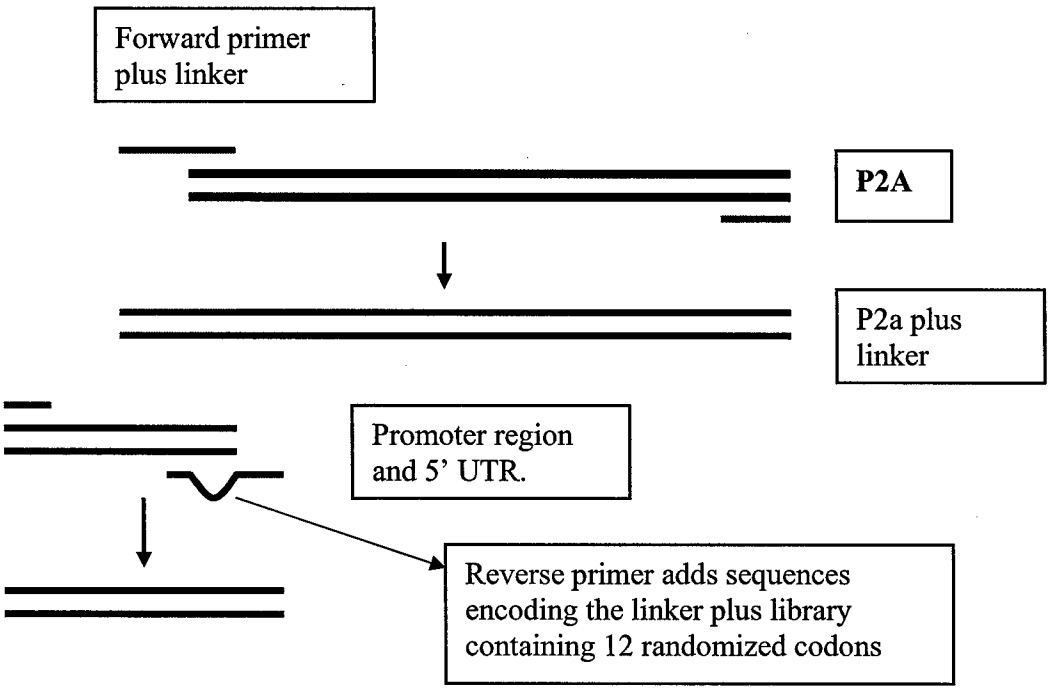
Figure 1 - Construction of 5 prime / amino terminus library:



Both fragments are gel purified and used to stitch the promoter-library and P2A together

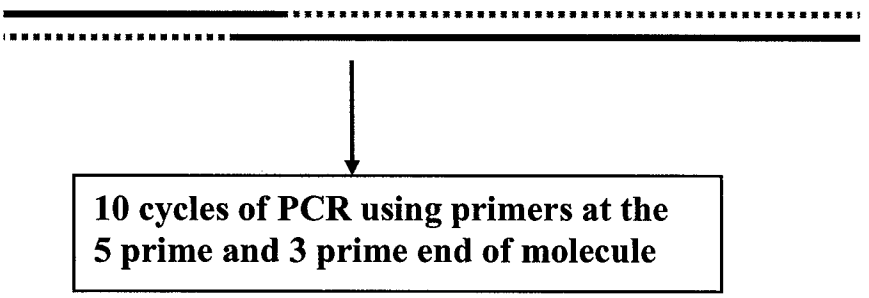


Figure 2 – Construction of the 3 prime library

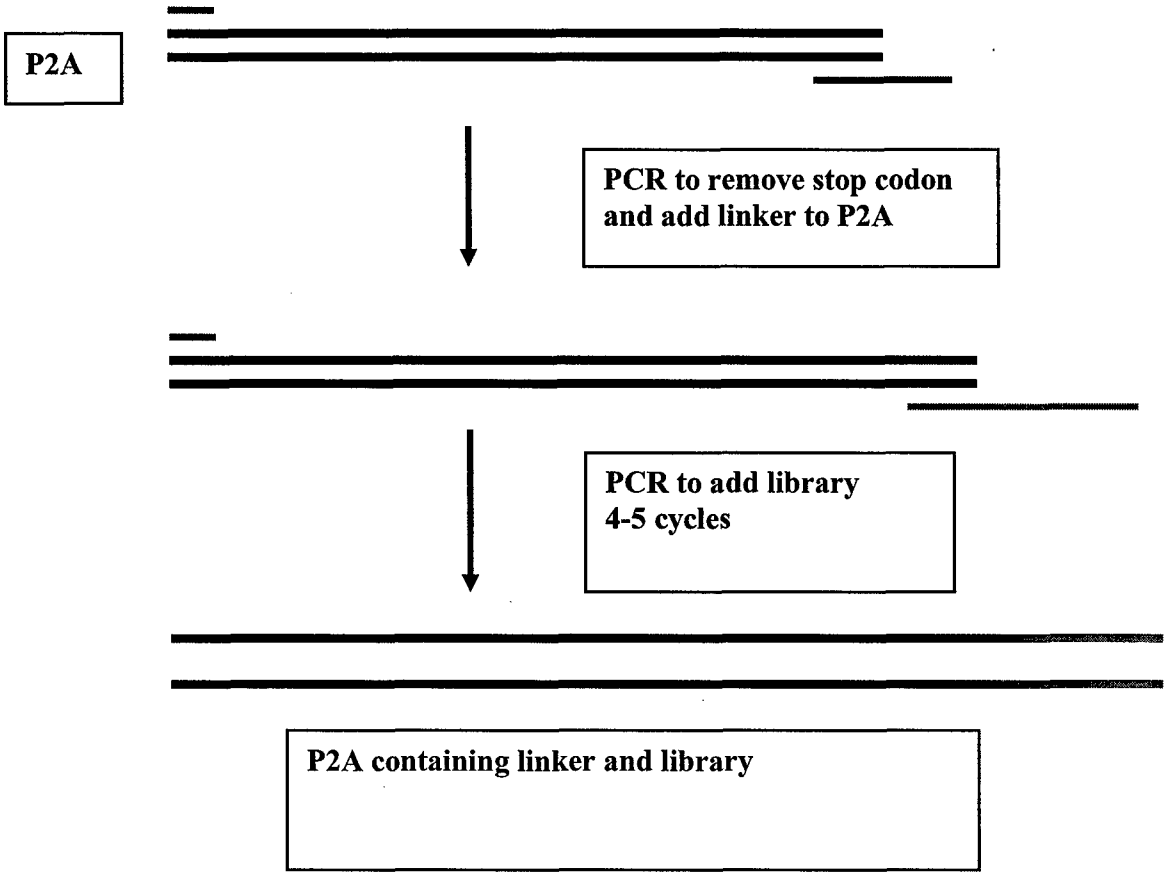
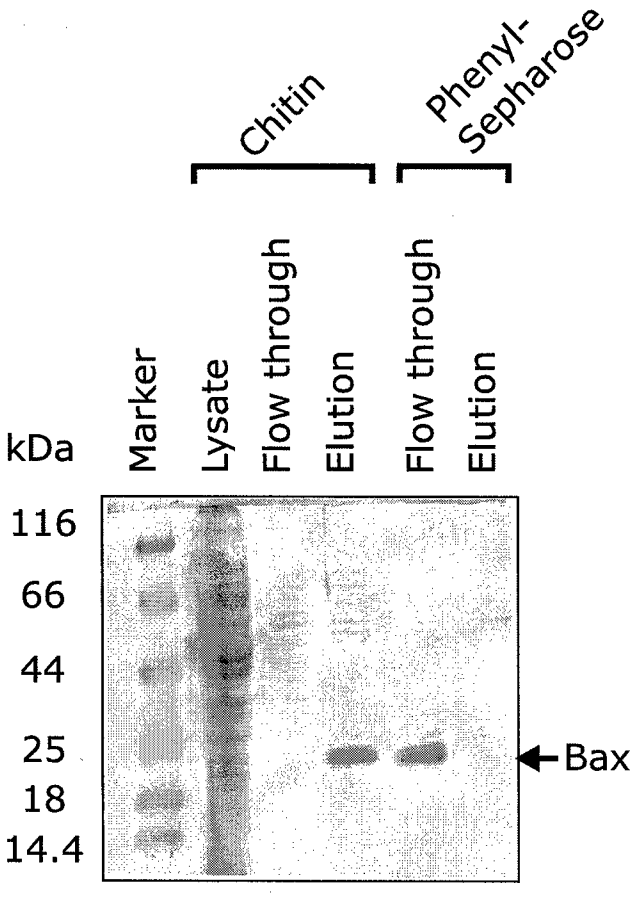
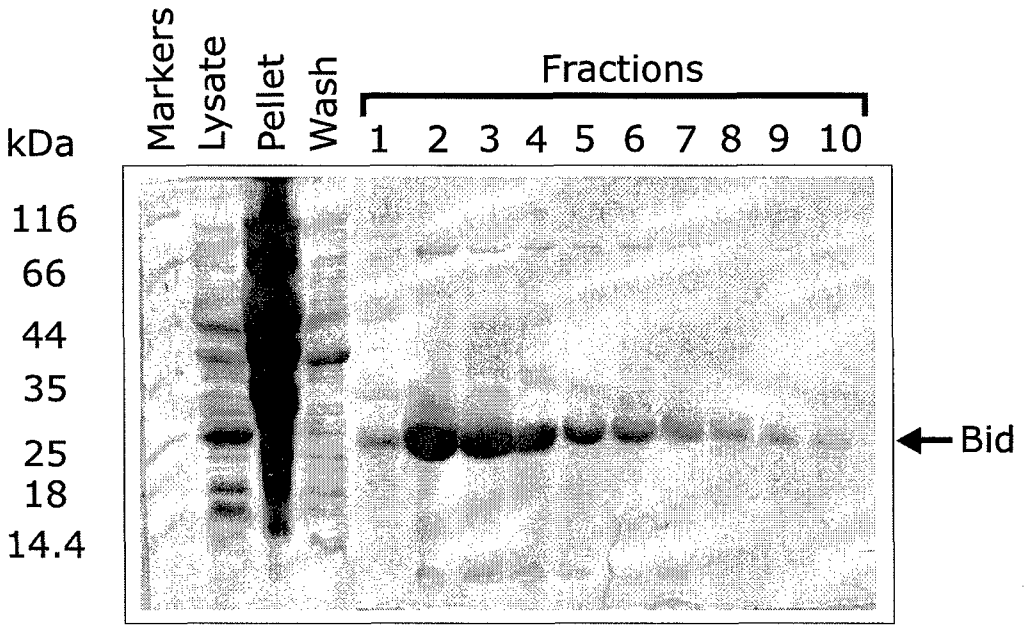


Figure 3 - Purification of Bax



Bax was expressed as an intein-chitin binding domain fusion protein. After binding to a chitin column intein cleavage was triggered by adding beta-mercaptoethanol. The eluted Bax was further purified by passing it through a phenyl-Sepharose column that binds some of the impurities while Bax passes through the column. Coomassie blue staining of an SDS-PAGE gel demonstrates efficient purification of Bax.

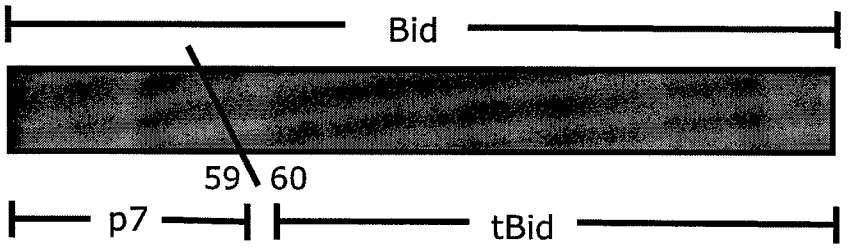
Figure 4 - Purification of Bid by metal ion chromatography



Bid with an amino-terminal His tag was expressed in BL21DE3 cells and purified on Nickel resin. The Coomassie blue stained SDS-PAGE gel of the fractions demonstrates purification of Bid.

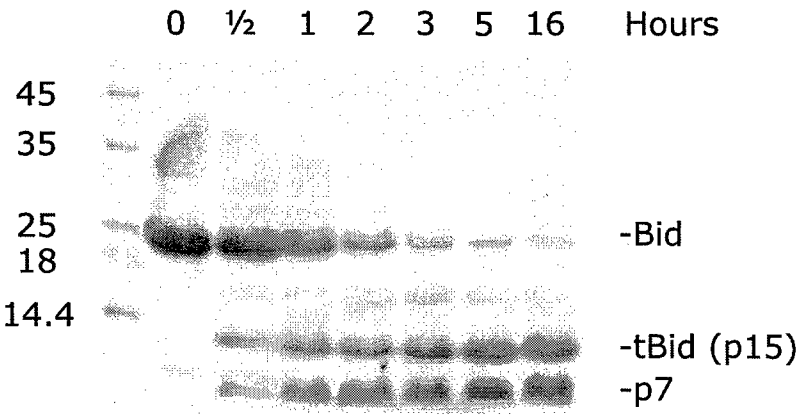
Figure 5 - Preparation of tBid from Bid

A)



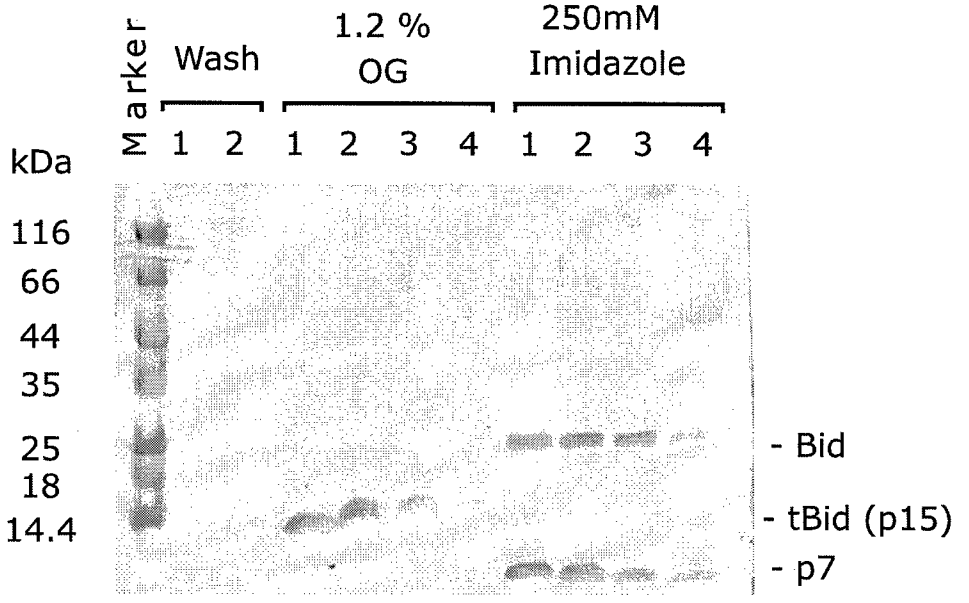
Bid must be cleaved using Caspase 8 to generate p7 and tBid

B)



Coomassie blue staining of an SDS-PAGE gel shows cleavage of Bid to tBid and p7 as a function of the time of incubation.

C)



Coomassie blue stained SDS-PAGE gel demonstrates isolation of tBid from p7 and residual uncleaved Bid by metal ion chromatography and elution with 1.2% octyl-glucoside (OG).

Figure 6. Conformation changes in Bax measured by fluorescence spectroscopy of Bax G138C.

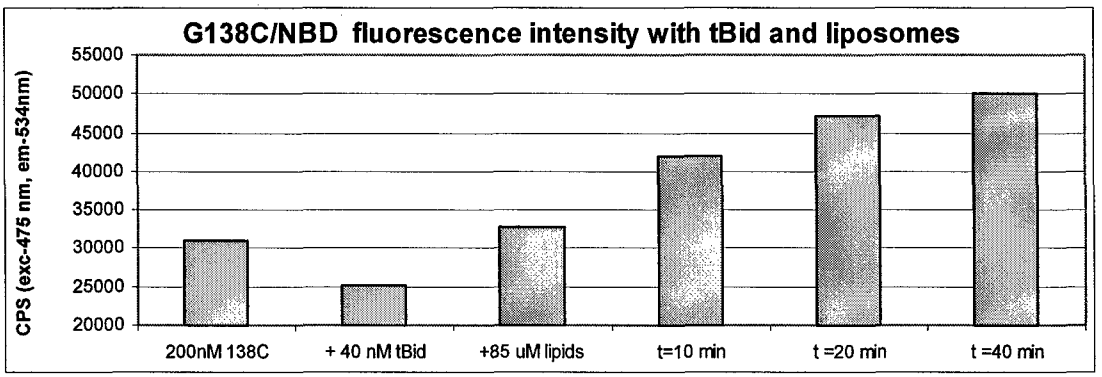


Figure 6: The NBD label on Bax G138C became more exposed to solvent when tBid bound Bax as seen by the decrease in fluorescence when 40 nM tBid was added. This indicates that helix 6 moves out of the protein interior when tBid binds. When liposomes are added to the reaction Bax inserts into the bilayer and the fluorescence intensity of the NBD label increased. 40nM tBid was added to 200 nM Bax, the fluorescence intensity was recorded and then liposomes with lipid composition similar to mitochondrial outer membrane were added and fluorescence intensity was measured over time. The incubation buffer was 100 mM NaCl, 10mM HEPES pH 7.4 and 0.2 mM EDTA. Separation of liposomes from the reaction revealed that without tBid no Bax was inserted into membranes yet when tBid was added all of the Bax inserted into membranes. Therefore, the starting population of Bax is all in the inactive conformation and all of it is converted to the active conformation by 40 nM tBid.

Figure 7A. Amino acid sequences of peptide sequences linked to P2A after one round of selection on native Bax and counter selection on Triton activated Bax. 9 codons are STOP codons compared to 12 expected without selection.

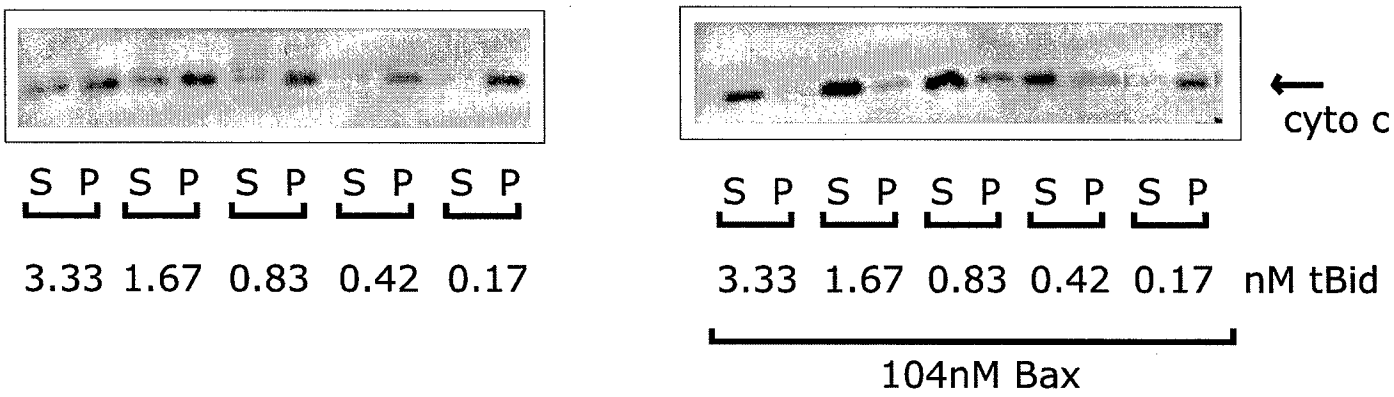
CLONE												
BT1	K	G	G	W	K	R	M	G	E	D	R	S
BT3	W	H	A	N	STOP	P	F	R	G	G	P	L
BT4	F	L	C	M	R	E	R	D	V	C	K	G
BT5	A	R	C	E	L	V	V	C	Q	P	G	G
BT6	R	M	L	Y	D	P	W	W	N	G	E	Y
BT7	S	H	N	G	C	S	C	R	STOP	S	R	L
BT8	I	A	G	D	K	R	G	S	R	D	G	A
BT9	R	I	H	L	R	I	T	F	R	G	P	G
BT10	C	L	V	W	V	P	I	Y	W	Y	STOP	S
BT11	P	G	K	R	K	W	V	STOP	Y	G	Q	S
BT13	E	G	R	W	R	V	E	R	STOP	Y	G	G
BT15	L	W	LN	Y	H	R	R	M	G	E	R	G
BT16	A	STOP	N	K	R	G	I	C	A	C	I	C
BT18	STOP	R	V	STOP	R	G	C	V	G	A	M	E
BT17	L	A	L	A	D	E	L	S	M	S	S	R
BT19	S	E	K	E	Y	K	R	Y	V	F	R	A
BT20	S	E	K	E	Y	K	R	Y	V	F	R	A
BT24	S	H	S	T	I	A	A	L	A	V	S	R
BT25	D	T	I	Y	C	T	S	S	G	M	A	D
BT27	S	H	D	G	C	S	C	R	STOP	S	R	L

Figure 7B. Amino acid sequences of peptide sequences linked to P2A after two rounds of selection on native Bax and one counter selection on Triton activated Bax. 9 codons are STOP codons compared to 15 expected without selection.

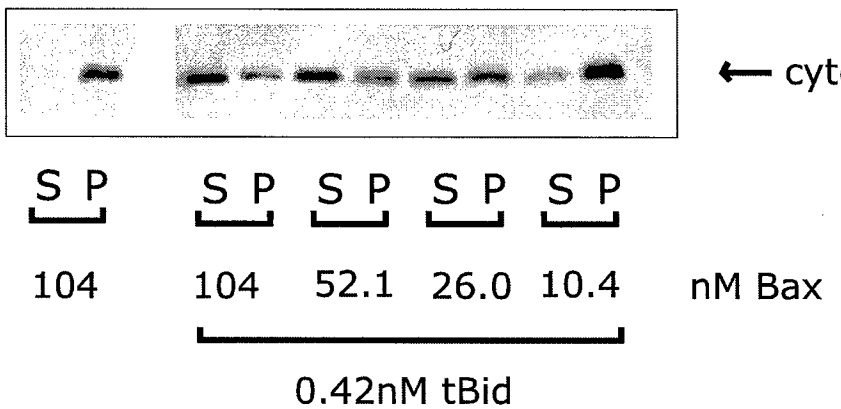
clone												
BTB9	C	H	A	M	H	T	K	Y	W	F	R	V
BTB10	A	D	M	S	N	F	C	STOP	R	S	H	F
BTB11	L	Q	N	Q	G	K	P	W	S	T	C	D
BTB12	N	V	L	L	P	T	D	R	W	G	A	N
BTB14	G	P	C or W	R	T	G	L	I	S	G	G	G
BTB15	STOP	L	H	E	G	S	G	V	V	Y	F	G
BTB16	STOP	Y	E	D	L	N	A	S	L	M	Q	G
BTB17	W	N	V	A	R	A	I	P	S	T	C	G
BTB20	H	G	I	R	E	E	S	L	E	L	L	S
BTB22	L	M	W	T	N	F	M	I	Q	G	STOP	G
BTB24	V	R	P	F	G	STOP	R	Y	L	W	C	A
BTB25	R	G	S	P	L	W	F	R	E	G	I	N
BTB27	A	R	G	E	R	L	C	R	G	R	C	S
BTB30	F	F	V	L	C	Y	R	A	G	R	W	N
BTB33	K	G	R	V	G	Q	G	STOP	M	L	Y	T
BTB35	STOP	I	F	H	V	T	S	D	G	E	A	A
BTB36	C	N	E	G	R	Y	N	K	L	F	T	S
BTB38	Y	M	Y	K	R	G	Q	W	G	Q	V	F
BTB40	L	L	S	I	L	V	F	STOP	V	M	G	A
BTB58	K	V	T	R	S	A	G	R	R	A	C	G
BTB59	T	Y	M	C	D	H	N	E	Q	W	P	G
BTB61	V	R	S	Y	S	G	N	G	A	P	N	G
BTB62	Y	G	L	STOP	A	V	E	K	A	A	G	G
BTB63	G	G	V	T	L	L	Q	C	V	K	Y	Y
BTB64	S	G	I	F	Q	F	K	E	K	R	K	R

Figure 8. An in vitro assay for Bax activity. Bax is activated to release cytochrome c by tBid

A) Cytochrome c release due to tBid alone is minimal. When Bax is present, tBid triggers it to release cytochrome c

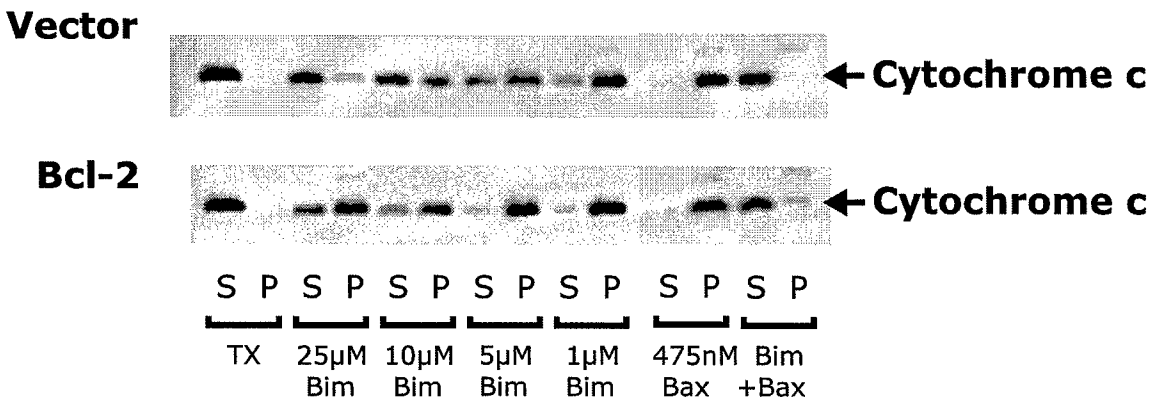


B) Cytochrome c release depends on the amount of Bax activated by tBid



To assay Bax activation and cytochrome c release, the specified concentrations of Bax and tBid were incubated with mitochondria isolated from Homyc3 cells. After incubation, mitochondria were pelleted by centrifugation (P) and cytocrhrome c released into the supernatant (S) was compared to that remaining in the pellet fractions by western blotting equivalent fractions of S and P fractions.

Figure 9.
Cytochrome c release induced by Bim BH3 peptide in vitro



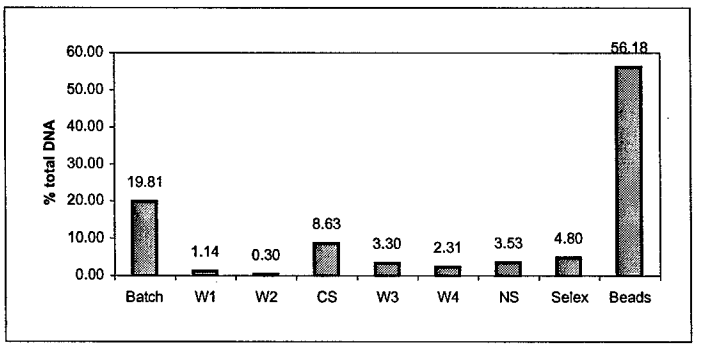
Mitochondria were prepared from cells transfected with a control vector (Vector) or with a plasmid encoding Bcl-2 (to increase resistance to activated Bax). Incubation with <10 uM Bim BH3 peptide caused minimal release of cytochrome c (particularly in mitochondria with Bcl-2). 475 nM Bax alone caused no release of cytochrome c. However activation of 475 nM Bax with 1 uM Bim peptide (Bim + Bax) caused efficient release of cytochrome c. This result is consistent with the recombinant Bax being in an inactive conformation until activated with BH3 peptide. Furthermore, this data demonstrates that a cell free system was established that can be used to assay aptamers to Bax (Task 2-b.).

Figure 10, Selection of ssDNA aptamers to conformers of Bax

Bax (native-Bax) Library = LAA
[protein] = 200 nM Selection round 4

Counter-selection = 200nM Bax plus 100uM Bid Bh3

background		30 minus bac	Percent
Batch	34612.07	34582.07	19.81
W1	2019.75	1989.75	1.14
W2	545.11	515.11	0.30
CS	15087.33	15057.33	8.63
W3	5783.63	5753.63	3.30
W4	4062.82	4032.82	2.31
NS	6195.78	6165.78	3.53
Selex	8413.13	8383.13	4.80
Beads	98088.27	98058.27	56.18
	total	174537.9	



Legend

Batch	DNA+biotin anti-sense incubated with avidin beads for 30 min pellet beads and count supernatent
Washes(W)	Beads are washed with buffer for 5 minutes pellet beads and count supernatent
Counter Selection (CS)	Beads are washed with counter-selection solution for 30 minutes pellet beads and count supernatent
Negative Selection (NS)	Beads are washed with buffer for 30 minutes pellet beads and count supernatent
Selection (Selex)	Beads are washed with selection solution for 30 minutes pellet beads and count supernatent
Beads	Resuspend beads in buffer and count

The recovery of slightly more aptamers when selected with Bax compared to the control (NS) suggests that selection of specific aptamers is occuring. Such a small increase in specific aptamers compared to control is normal for round 4 of selection. The counter selection released even more specific aptamers, as expected since counter selection precedes specific selection. That most of the DNAs remained bound to beads is also expected at early rounds of selection.

Figure 11 Summary of the last 2 rounds of selection of aptamers to different Bax conformers (Replacement Task 3)

Legend

Bax A	Selected for soluble Bax and against soluble Bax plus Bid BH3
Bax B	Selected for soluble Bax plus Bid BH3 and against soluble Bax, as well as peptide alone
Bax C	Selected for membrane Bax (protein,peptide,liposomes) and against soluble Bax, as well as peptides and liposomes
Bax D	Selected for soluble Bax and against membrane Bax (protein,peptide,liposomes)

The CPM data is given for the Negative Selection (buffer wash) and the subsequent selection

Relative numbers are simply Negative Selection CPM / Selection CPM (>1 means more DNA was released from beads in the presence of selected protein than from the buffer alone)

BaxA	CPM round 5	Relative	CPM round 6	Relative
Negative Selection	154.03	1	2048.92	1
Selection	214.56	1.392975	14523.15	7.088198

BaxB	CPM round 4	Relative	CPM round 5	Relative
Negative Selection	112.06	1	2696.93	1
Selection	170.6	1.522399	12509.15	4.638292

BaxC	CPM round 3	Relative	CPM round 4	Relative
Negative Selection	37.54	1	2135.03	1
Selection	25.53	0.680075	9528.55	4.462958

BaxAb	CPM round 4	Relative	CPM round 5	Relative
Negative Selection	1560.32	1	11445.82	1
Selection	2104.95	1.34905	11628.41	1.015953